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# Cystatin C in adipose tissue and stimulation of its production by growth hormone and triiodothyronine in 3T3-L1 cells

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ABSTRACT

Cystatin C (CysC) is a marker for estimation of glomerular filtration rate (GFR). CysC levels may depend not only on clearance/GFR but possibly also on changes in production. Our studies on tissue distribution of CysC protein in mice showed that adipose tissue expresses significant amounts of CysC, suggesting that adipocytes could contribute to circulating CysC levels *in vivo*. As growth hormone (GH) and triiodothyronine ($T_3$) increase both GFR and CysC (increased in acromegaly and hyperthyroidism) *in vivo*, we studied whether they could increase CysC production in 3T3-L1 adipocytes *in vitro*. CysC accumulated in culture media of 3T3-L1 adipocytes in a time-dependent fashion. GH and $T_3$ both (10 nmol/l) increased accumulation of CysC, to 373 ± 14 and 422 ± 20, respectively, vs 298 ± 10 ng per well over 4 days in controls. Thus, GH and $T_3$ enhance the production of CysC by adipocytes *in vitro*.

## 1. Introduction

Cystatin C (CysC) is a basic protein of 13'300 Da and a competitive endogenous inhibitor of papain and related lysosomal cysteine proteinases. CysC is an excellent indicator of glomerular filtration rate (GFR) as it is released by most cells at a constant rate (Abrahamson et al., 1990; Cole et al., 1989) and cleared from the bloodstream by the kidneys; it is filtered by glomeruli and subsequently catabolized by tubular epithelial cells. CysC concentration is negatively correlated with GFR (Simonsen et al., 1985) and it is used as an alternative marker to creatinine for estimation of GFR. However, plasma concentrations of endogenous filtration markers can be influenced by factors other than GFR (non-GFR determinants), such as changes in production rate, renal tubular reabsorption/secretion and extrarenal elimination. CysC is considered superior to creatinine (predominantly produced in muscle) as a marker for detection of mild renal dysfunction (in the "creatinine-blind" range); moreover, CysC is an interesting biomarker associated with aging, cardiovascular risk, frailty, and overall mortality (Astor et al., 2012; Ballew et al., 2017; Emberson et al., 2010; Hart et al., 2017; Ix et al., 2007; Koenig et al., 2005; Newman et al., 2016; Sarnak et al., 2008; Shlipak et al., 2005; Tangri et al., 2012; Woitas et al., 2013; Yamashita et al., 2013), particularly in elderly and in obese patients. The latter may be due not just to differences in renal handling of creatinine and CysC, but also due to differences in tissue origin and production rates of the two compounds. Whereas the muscular origin of creatinine is well documented, the relative contribution of several organs to circulating levels of CysC is unknown and difficult to estimate. Adipose tissue may contribute significantly to elevated serum CysC (Naour et al., 2009). CysC has been found to increase with obesity (Naour et al., 2009; Chew-Harris et al., 2013; de Boer et al., 2012; Muntner et al., 2008; Shankar and Teppala, 2011; Young et al., 2008; Stevens et al., 2009), to rise in proportion to the number of metabolic syndrome components, and to be associated with insulin resistance and endothelial dysfunction (Lee et al., 2010; Murai et al., 2017; Servais et al., 2008; Surendar et al., 2010; Vigil et al., 2009). Increased serum levels of CysC have been found indicative of the metabolic syndrome and type 2 diabetes (Donahue et al., 2007; Magnusson et al., 2013; Reutens et al., 2013; Sahakyan et al., 2011). Interestingly, this relationship between levels of serum CysC and insulin resistance was found not only in type 2 but also in type 1 diabetes (Uruska et al., 2014). Although CysC can be considered as a marker and predictor of insulin resistance (de Boer et al., 2012; Lee et al., 2010; Servais et al., 2008; Surendar et al., 2010; Vigil et al., 2009; Codoner-Franch et al., 2011; Lee et al., 2009), it remains unclear whether and to what extent this epidemiological association is based on altered adipose tissue mass and/or function (Naour et al., 2009; Naour et al., 2010), e.g. in the context of inflammation of adipose tissue or of hyperinsulinemia. CysC appears to play an important role in adipose tissue remodelling. It

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inhibits the protease activity of cathepsin S, a biomarker for adiposity that is expressed by adipose tissue and up-regulated in obesity. Cathepsin S degrades several extracellular matrix (ECM) proteins, including fibronectin. ECM proteins are downregulated and CysC expression is upregulated during the process of adipocyte differentiation (Kratchmarova et al., 2002; Taleb et al., 2005).

Tissue-specific expression of CysC has been studied in mice. Huh et al. found that CysC mRNA expression was especially high in brain and testis, and low in liver and pancreas (Huh et al., 1995). Hakansson (Hakansson et al., 1996) and Jiborn (Jiborn et al., 2004) measured CysC by immunoassay in various mouse tissues and found high abundance in brain and low abundance in liver; the abundance was intermediate in skeletal muscle. These authors did not comment on adipose tissue although this tissue may be a relevant source contributing to circulating CysC.

Non-GFR determinants of CysC have been inferred mainly from cross sectional studies and remain poorly defined (Levey et al., 2014). Besides abdominal obesity and insulin, there are specific endocrine disorders that may affect CysC levels independently of GFR. Hypothyroidism and hypopituitarism are associated with lower CysC serum levels that increase upon thyroid hormone replacement therapy (Fricker et al., 2003; Goede et al., 2009). Remarkably, growth hormone (GH) or thyroid hormone excess (acromegaly and hyperthyroidism) both lead to concomitant increases in GFR and CysC levels, which can be reversed following successful treatment (Fricker et al., 2003; Sze et al., 2013). Thyroid dysfunction and acromegaly lead to discrepancies in CysC and creatinine levels (Fricker et al., 2003; Goede et al., 2009; Sze et al., 2013; den Hollander et al., 2003; Jayagopal et al., 2003; Kotajima et al., 2010; Manetti et al., 2005; Wiesli et al., 2003). Thyroid hormones and GH may increase the production of CysC so that its levels could increase despite an increased clearance of creatinine and an elevated GFR.

3T3-L1 cells (Green and Meuth, 1974) have been used to study differentiation of adipocytes *in vitro*. Differentiation of these cells to adipocytes can be monitored by assessing preadipocyte factor-1 (Pref-1). Pref-1 was cloned from 3T3-L1 cells (Smas and Sul, 1993) and is highly expressed in proliferating adipocyte-lineage committed fibroblasts/preadipocytes but absent in differentiated adipocytes. Proteomic approaches for identification of proteins expressed and secreted by adipocytes have found CysC as a prominent protein that is upregulated with differentiation, and increasingly secreted in response to insulin (Kratchmarova et al., 2002; Molina et al., 2009; Zhou et al., 2009). Furthermore, it has been found that $T_3$ stimulates glucose uptake in 3T3-L1 adipocytes in long term culture, as also in a rat liver cell line, in cardiomyocytes, and in preosteoblasts (Gosteli-Peter et al., 1996; Lin and Sun, 2011; Romero et al., 2000; Weinstein et al., 1990; Zoidis et al., 2012). Classical insulin target tissues such as liver, striated muscle, and adipose tissue may be particularly responsive to GH. Indeed, 3T3-L1 fibroblasts and adipocytes have been used to test effects of GH on differentiation and glucose uptake, respectively (Frost and Lane, 1985; Green et al., 1985; Stewart et al., 2004), and to study insulin-antagonistic effects of GH (Sasaki-Suzuki et al., 2009; Takano et al., 2001). By activating the JAK-STAT pathway, GH plays an important role in regulating adipogenesis and adipose tissue function (Richard and Stephens, 2014).

We studied tissue distribution of CysC in mice, including adipose tissue. We could confirm previous results showing high expression in pituitary and brain, and low expression in liver. In addition, we found significant expression of CysC in adipose tissue depots. The main goal of our study was to test if GH and $T_3$ directly regulate CysC production in adipocytes. Since it is difficult to exclude indirect effects *in vivo*, we decided to perform experiments *in vitro*. We chose the 3T3-L1 preadipocyte/adipocyte model, an adipocytic cell line that is well characterized, and is responsive to both of these hormones. We confirmed that GH and insulin stimulate phosphorylation of Stat5 and Akt/PKB in 3T3-L1 cells after several days of serum deprivation. We then studied whether GH and $T_3$ regulate the production of CysC, and we compared their effects with those of dexamethasone.

## 2. Materials and methods

### 2.1. Materials

Cell culture media, fetal calf serum (FCS), penicillin/streptomycin, glutamine, and trypsin were purchased from Thermo Fisher (Switzerland). Fatty acid-free bovine serum albumin (BSA) was from Sigma (Darmstadt, Germany). Dexamethasone (dex) was from Sigma and dissolved as a stock solution of 1 mmol/l in absolute ethanol. Triiodothyronine ($T_3$, 3,3′,5-triiodo-l-thyronine, sodium salt) was purchased from Sigma (St. Louis MO, USA), and, prior to use, dissolved as a stock solution of 1 mmol/l in 10 mmol/l NaOH. Isomethylbutylxanthine (IBMX) was from Sigma, GH and human insulin were kindly provided by Novo Nordisk (Gentofte, Denmark). 2-deoxy-D-[1–$^{14}$C] glucose (0.2 μCi/ml; 58 mCi/mmol) was from Perkin Elmer (Waltham MA, USA).

### 2.2. Isolation of tissues from mice

Wild type mice (C57BL/6) on standard chow diet (Provimi Kliba SA, Kaiseraugst, Switzerland) were housed in groups of up to five littermates with 12 h dark-light cycle at 21 °C and were allowed free access to food and water. Breeding of mice and all conducted procedures were carried out in agreement with Swiss animal protection laws and were approved by the appropriate authorities. 52 days-old mice were sacrificed and their organs dissected. Tissues were homogenized in 50 mmol/l HEPES pH 7.5, 140 mmol/l NaCl, 1 mmol/l PMSF, 0.5% Triton X-100, 10 mmol/l NaF, 1 mmol/l $Na_2H_2P_2O_7$, 1 mmol/l $Na_2O_4V$, 3 μg/ml aprotinin, 3 μg/ml leupeptin. Protein concentration in cleared lysates was determined by the bicinchoninic acid (BCA) protein assay kit from Pierce (USA, Rockford IL). As protein yield was low from adipose tissue and endocrine organs, tissues were pooled from four animals. Homogenates were stored at −70 °C until used.

### 2.3. Cell culture

3T3-L1 cells (a mouse preadipocytic cell line, purchased from ATCC, Manassas, USA) were cultured and passaged in TPP tissue culture flasks (Trasadingen, Switzerland) in Dulbecco's modified Eagle's medium (DMEM) containing 25 mmol/l glucose. 10% (v/v) FCS, 1% penicillin, 1% streptomycin, and 2 mmol/l L-glutamine. The cells were kept at 37 °C in an atmosphere of 5% v/v $CO_2$ in air.

As shown in Fig. 1, cells were plated and cultured under conditions initiating differentiation of 3T3-L1 fibroblasts into adipocytes. Cells were seeded (12'500 cells/cm$^2$) on gelatinized plates and cultured in growth medium for 3 days with high D-glucose DMEM containing FCS (10%), L-glutamine, and penicillin/streptomycin as described above. For the next two days (3–5) the DMEM contained 2% FCS, L-glutamine, penicillin/streptomycin and differentiation mix (1.7 μmol/l insulin, 1 μmol/l dex, 0.5 mmol/l IBMX and 1 μmol/l rosiglitazone). Cells were washed with PBS and kept in serum-free DMEM, containing 1 g/l BSA, glutamine, penicillin/streptomycin, 100 nmol/l dex, and 10 nmol/l insulin for two days (5–7). Cells were washed with PBS prior to culture in serum-free DMEM, containing 1 g/l BSA, glutamine, penicillin/streptomycin for the last four days (7–11). Hormones or corresponding vehicles were directly added to the media, unless indicated otherwise. Supernatants were collected and cells were lysed after 11 days of culture.

### 2.4. Assessment of 2-deoxyglucose uptake (glucose transport)

Cells were grown in multiwell tissue culture dishes (35 mm diameter). Insulin-dependent uptake of 2-deoxy-D-[1–$^{14}$C] glucose (2DG)

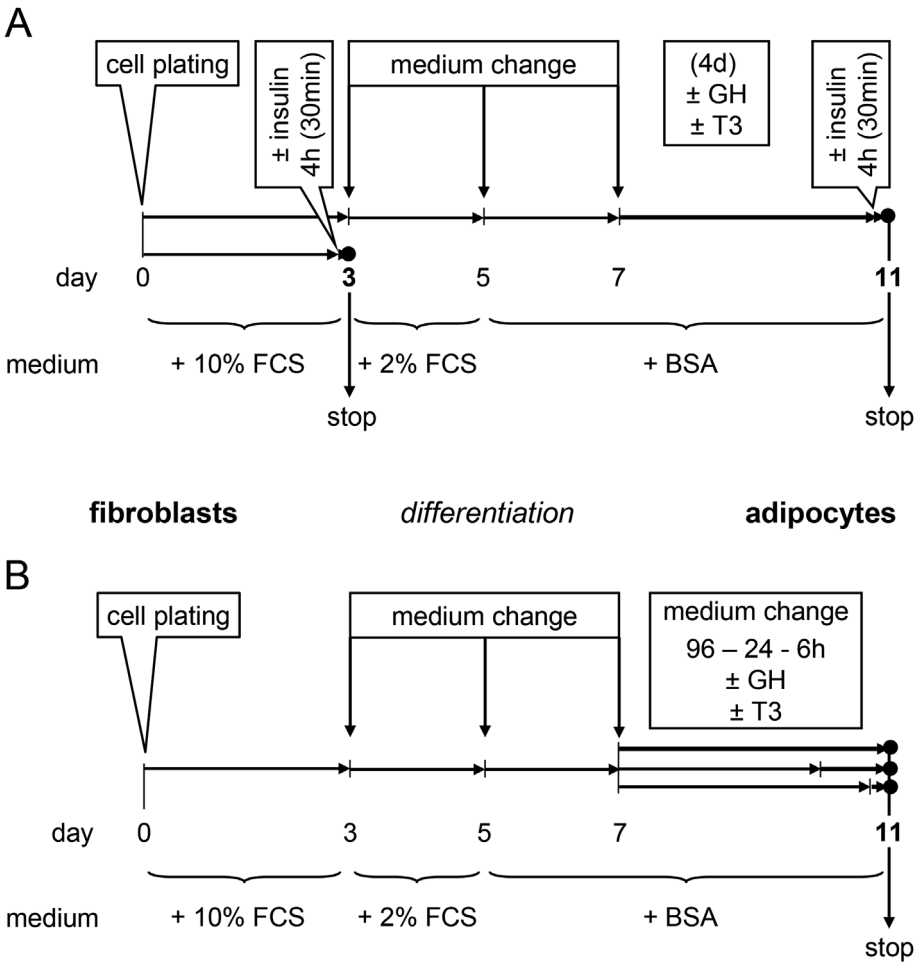


**Fig. 1.** Flow diagram (time course) of cell treatment protocols.
Cells were cultured for 3 or 11 days. In the latter case, the last 6 days were without serum. GH, $T_3$, dex, or insulin were added as indicated. Cells were characterized and Insulin responsiveness was assessed as shown in A. Fibroblasts/preadipocytes after 3 days and adipocytes after 11 days. For long-term incubations with GH or $T_3$, two distinct protocols were used (**A** and **B**). Protocol B includes media replacement at different time points to assess time-dependent hormone effects and accumulation of CysC in culture media as shown in Fig. 4.

was allowed over 10 min at room temperature as described previously (Zoidis et al., 2012). In brief, monolayers were washed at room temperature with 1 ml transport buffer containing 140 mmol/l NaCl, 5 mmol/l KCl, 1 mmol/l $MgCl_2$, 1.5 mmol/l $CaCl_2$, and 15 mmol/l N-2-hydroxyethylpiperazine- N-2-ethanesulfonic acid (HEPES, adjusted to pH 7.4 with Tris-HCl). Uptake studies were initiated by adding 1 ml of transport buffer containing 0.1 mmol/l cold 2DG and [1–$^{14}$C]2DG (0.2 µCi/ml). Ten minutes later, the buffer was removed and the dishes were quickly rinsed three times with 1 ml of ice-cold transport buffer. The cells were then solubilized with 1 ml of 2% w/v sodium dodecyl sulfate (SDS), and the radioactivity of a 0.5 ml aliquot was measured in a liquid scintillation counter.

Protein content was determined in parallel in identically treated dishes. After 11 days of culture (of which six days in serum-free medium and the last four days in test medium), cells from parallel dishes were lysed into 1 ml of 0.1% w/v Triton X-100 for the determination of protein content by a modification of the Lowry procedure or by the bicinchoninic acid (BCA) method (Pierce, Rockford IL, USA) as described (Zoidis et al., 2012).

### 2.5. RNA extraction and quantitative RT-PCR

Total RNA was extracted from 3T3-L1 fibroblasts or adipocytes (grown on 12-well plates, 50'000 cells seeded per 3.5 $cm^2$) using the NucleoSpin® RNA Kit (Macherey-Nagel). Concentration and purity of the extracts were analysed with a NanoDrop Lite spectrophotometer (Thermo Scientific). RNA (0.30 µg) was reverse transcribed with Superscript III Reverse Transcriptase (Thermo Fisher) using random hexamer primers (Thermo Fisher). A 7500 FAST Real-Time PCR system (Applied Biosystems) was used for real-time PCR amplification. Relative gene expression was obtained after normalization to 18s RNA, using formula $2^{-\Delta\Delta cp}$. The following primers (Applied Biosystems) were used: Mm00438347_m1 for *cysC* mRNA and Mm03928990_g1 for 18s ribosomal RNA.

### 2.6. Immunoblotting

Cells were rinsed and lysed in 50 mmol/l HEPES pH 7.5, 140 mmol/l NaCl, 1 mmol/l PMSF, 0.5% Triton X-100, 10 mmol/l NaF, 1 mmol/l $Na_2H_2P_2O_7$, 1 mmol/l $Na_2O_4V$, 3 µg/ml aprotinin, 3 µg/ml leupeptin. Protein concentration in cleared lysates was determined by the bicinchoninic acid (BCA) protein assay kit from Pierce (USA, Rockford IL). Equal amounts of protein (tissue homogenate or cell lysate) were separated by SDS-PAGE (NuPAGE, Thermo Fisher, Switzerland) and transferred onto nitrocellulose membranes. 2% non-fat milk in TBST (10 mmol/l Tris-HCL, pH 7.4, 150 mmol/l NaCl, 0.05% (v/v) Tween 20) was used to block nonspecific binding of antibodies to membranes. Incubation with primary and secondary antibodies was either at room temperature for 1 h or overnight at 4 °C. Equal loading and transfer was confirmed by Ponceau S staining and with an antibody against actin

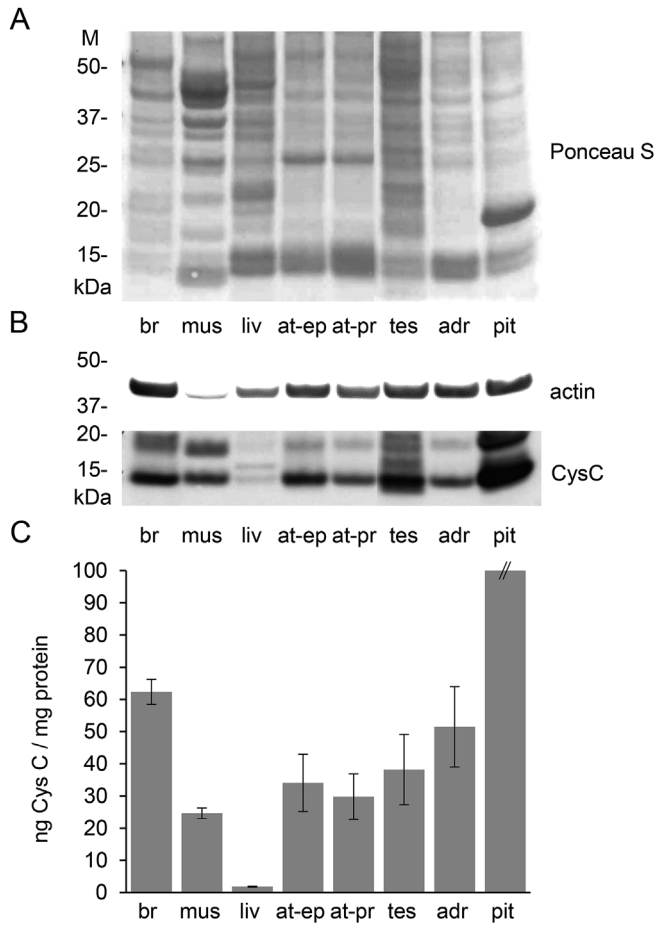


**Fig. 2.** Tissue-specific expression of CysC in mice.
Tissue extracts from male mice were loaded onto SDS-PAGE and blotted to nitrocellulose membranes. Proteins were stained by Ponceau S (**A**). Actin and CysC were assessed with specific antibodies (**B**). To quantify CysC protein, the same extracts were analysed by ELISA (**C**); results are shown as mean ± SEM. The following tissues were analysed: Brain (br), skeletal muscle (mus), liver (liv), epididymal adipose tissue (at-ep), perirenal adipose tissue (at-pr), testis (tes), and adrenal (adr) and pituitary (pit) gland.

**Table 1**
CysC expression in 3T3-L1 fibroblasts and adipocytes.

Cells were cultured for 3 days or 11 days as outlined in Fig. 1A. mRNA expression of CysC was assessed by quantitative RT-PCR and is presented normalized for day 3 and to 18S RNA, 2–ΔΔcp (n = 2 in duplicate). Abundance of CysC protein in cell layers normalized to day 3 was assessed by densitometric analysis of Western blots (n = 3 in duplicate).

| cystatin C | d3 (fibroblasts) | d11 (adipocytes) |
|---|---|---|
| mRNA | (1) | 2.37 ± 0.39 |
| protein | (1) | 3.04 ± 0.38 |

(MAB1501: EMD Millipore, Temecula, USA). GH- and insulin-dependent signaling was assessed with antibodies against the phosphorylated forms of Stat5a/b (Tyr 694): Santa Cruz, Dallas, USA and Akt/PKB (Ser473): Cell Signaling, Danvers, USA and ERK1/2 (Thr202/Tyr204): Cell Signaling, Danvers, USA. For analysis of CysC, cystatin C (rabbit polyclonal) antiserum was from DAKO (Glostrup, Denmark), the secondary antibody, goat anti-rabbit HRP, from Bio-Rad (Reinach, Switzerland); both were used at a dilution of 1:3000. Pref-1/DLK1/FA1 antibody (goat polyclonal) was from R&D Systems (Minneapolis, USA) and used at a dilution of 1:1000; the secondary antibody (donkey anti-goat) was from Santa Cruz (Dallas, USA). PPARγ antibody was from

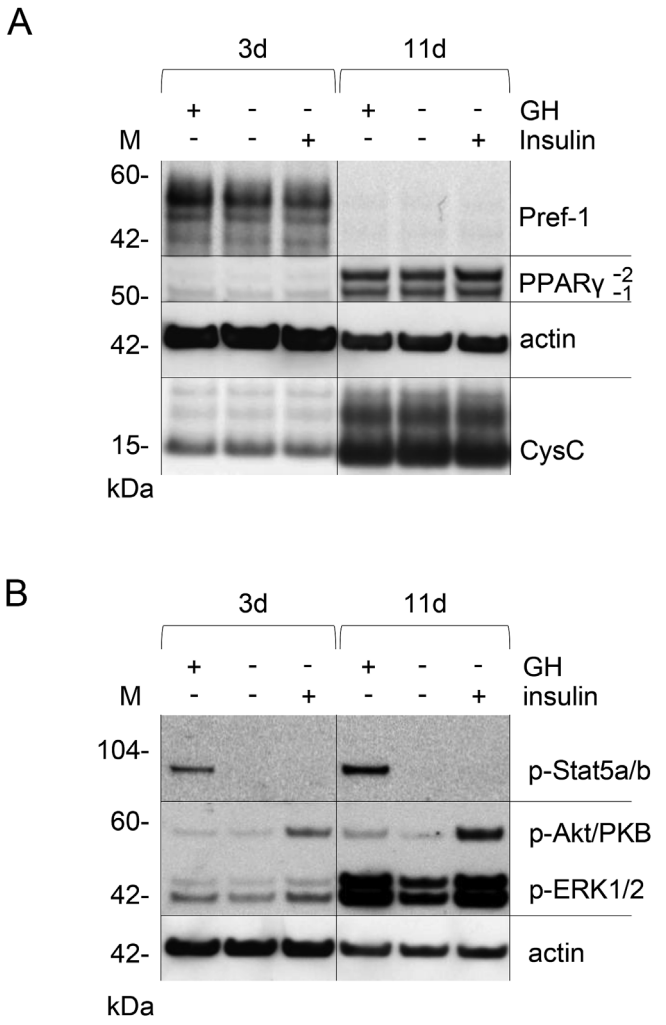


**Fig. 3.** Hormone responsiveness of 3T3-L1 cells and expression of Pref-1, PPARγ and CysC.
Western analysis of Pref-1, PPARγ and CysC (**A**) and p-Stat5a/b, p-Akt/PKB, and p-ERK1/2 (**B**) in fibroblasts/preadipocytes (3d of culture) and in adipocytes (11 d in culture) exposed to 10 nM GH or 100 nM insulin for 30 min. Actin was visualized as a control. Cells were kept on dishes for 3 days or 11 days as outlined in Fig. 1A, and exposed to medium containing hormones for the last 30 min.

Santa Cruz (Dallas, USA) and used at a dilution of 1:1000. Immunoreactive proteins were visualized by the Lumi-Light Western Blotting Substrate (Roche) using a LAS-3000 imaging system (Fuji). Signal intensities were quantified using the AIDA software package from Raytest (Germany).

### 2.7. ELISA for cystatin C

Cell culture media were collected as described above and analysed by a sandwich enzyme immunoassay for quantitative measurement of mouse CysC (BioVendor, Modrice, Czech Republic). The polyclonal antibodies have been raised against mouse CysC that was also used for the calibration curve. Determination of CysC in mouse tissue extracts was as described for cell culture media.

### 2.8. Statistical analysis

Results were obtained by pooling data from independent experiments in which the same conditions were tested, as indicated. Data are expressed as means ± S.E.M. Statistical significance was checked by

**Table 2**
Effects of GH, $T_3$, and dex on 2DG uptake.

Cells were cultured for 11 days and exposed to GH [10 nM] or $T_3$ [10 nM] (**A**) or dex [100 nM] (**B**) for the last 4 days of culture; the medium was changed after 92 h and insulin was included in the test medium for the last 4 h (protocol as in Fig. 1A). 2DG uptake was measured over a period of 10 min. Values are mean ± SEM from at least 5 experiments in triplicate; *p < 0.05 for comparison of GH or $T_3$ vs control. ns for comparison of dex vs control.

A

| | 2DG uptake (pmol/mg protein x 10 min) | | |
|---|---|---|---|
| | insulin [nM] | | |
| | 0 | 1 | 100 |
| Control | 232 ± 20 | 379 ± 37 | 836 ± 44 |
| GH [10 nM] | 221 ± 23 | 293 ± 3 | 641 ± 52* |
| $T_3$ [10 nM] | 364 ± 40* | 576 ± 53* | 1048 ± 59* |

B

| | 2DG uptake (pmol/mg protein x 10 min) | | |
|---|---|---|---|
| | insulin [nM] | | |
| | 0 | 1 | 100 |
| Control | 282 ± 33 | 479 ± 71 | 898 ± 117 |
| dex [100 nM] | 373 ± 22 | 549 ± 45 | 868 ± 56 |

paired Student's t-test or ANOVA, as appropriate. A *P*-value < 0.05 was considered to be statistically significant.

## 3. Results

### 3.1. Tissue distribution of CysC in mice

Tissue-specific expression of CysC in mice was assessed in organ protein extracts by Western blotting and ELISA (Fig. 2).

Immunoblots revealed significant differences in CysC expression among tissues analysed and a representative blot is shown in Fig. 2A and B. CysC was most abundant in the pituitary gland and lowest in liver. Intermediate expression was observed in brain, muscle, adipose tissue, testis and ovaries (not shown), and the adrenal gland.

Results obtained by ELISA correspond well with those from immunoblotting (Fig. 2C and B). We analysed males and females separately, however, only minor differences were observed. Pituitary contained the highest levels of CysC (> 100 ng/mg protein) and liver extracts the lowest (1.8 ± 0.5 ng/mg protein). Significantly higher levels than in liver were found in brain (62 ± 11 ng/mg protein), muscle (25 ± 5 ng/mg protein), adipose tissue depots (perigonadal, perirenal, and subcutaneous, on average, 39 ± 7 ng/mg protein), testis (38 ± 15 ng/mg protein), ovaries (17 ± 5 ng/mg protein), and adrenal gland (52 ± 13 ng/mg protein). Serum concentration of CysC (mean ± SD) was not different in male (628 ± 136 ng/ml, n = 8) and in female (586 ± 107 ng/ml, n = 8) animals.

### 3.2. Hormone responsiveness and expression of CysC in 3T3-L1 cells

High expression levels of CysC in adipose tissue from mice suggested that adipocytes could indeed contribute to alterations in circulating CysC levels *in vivo*.

First, expression of CysC was assessed at the RNA and protein levels in 3T3-L1 cells after 3 and 11 days of culture. As shown in Table 1, more

**Table 3**
Effects of GH, $T_3$, and dex on CysC production.

Cells were exposed to GH [10 nM] or $T_3$ [10 nM] (**A**) or dex [100 nM] (**B**) for the last 4 days of culture as shown in Fig. 1A and the 96 h time point of Fig. 4. Media (supernatant) as well as cell layers (protein extracts) were analysed by both ELISA and immunoblotting. As shown here and also stressed in the legend to Fig. 4, much more CysC protein accumulates over 4 days in the media (ELISA, n = 11; immunoblot, n = 6) than in the cell layers (ELISA, n = 4; immunoblot, n = 4). *p < 0.05 for comparison of hormone-treated vs control.

A

| | cystatin C | | | |
|---|---|---|---|---|
| | cell layer | | supernatant | |
| | ELISA ng/well x 4 d (relative to control) | Immunoblot densitometry (relative to control) | ELISA ng/well x 4 d (relative to control) | Immunoblot densitometry (relative to control) |
| control | 17.1 ± 1.8 (1) | (1) | 298 ± 10 (1) | (1) |
| GH [10 nM] | 28.6 ± 8.3 (1.46 ± 0.28) | (1.10 ± 0.03) | 373 ± 14* (1.25 ± 0.03)* | (1.18 ± 0.09) |
| $T_3$ [10 nM] | 32.8 ± 5.7 (1.79 ± 0.16)* | (1.37 ± 0.05) | 422 ± 20* (1.41 ± 0.04)* | (1.22 ± 0.06) |

B

| | cystatin C | | | |
|---|---|---|---|---|
| | cell layer | | supernatant | |
| | ELISA ng/well x 4 d (relative to control) | Immunoblot densitometry (relative to control) | ELISA ng/well x 4 d (relative to control) | Immunoblot densitometry (relative to control) |
| control | 17.4 ± 1.6 (1) | (1) | 241 ± 20 (1) | (1) |
| dex [100 nM] | 22.0 ± 2.5 (1.23 ± 0.08)* | (1.34 ± 0.10) | 283 ± 19 (1.22 ± 0.09)* | (1.17 ± 0.10) |

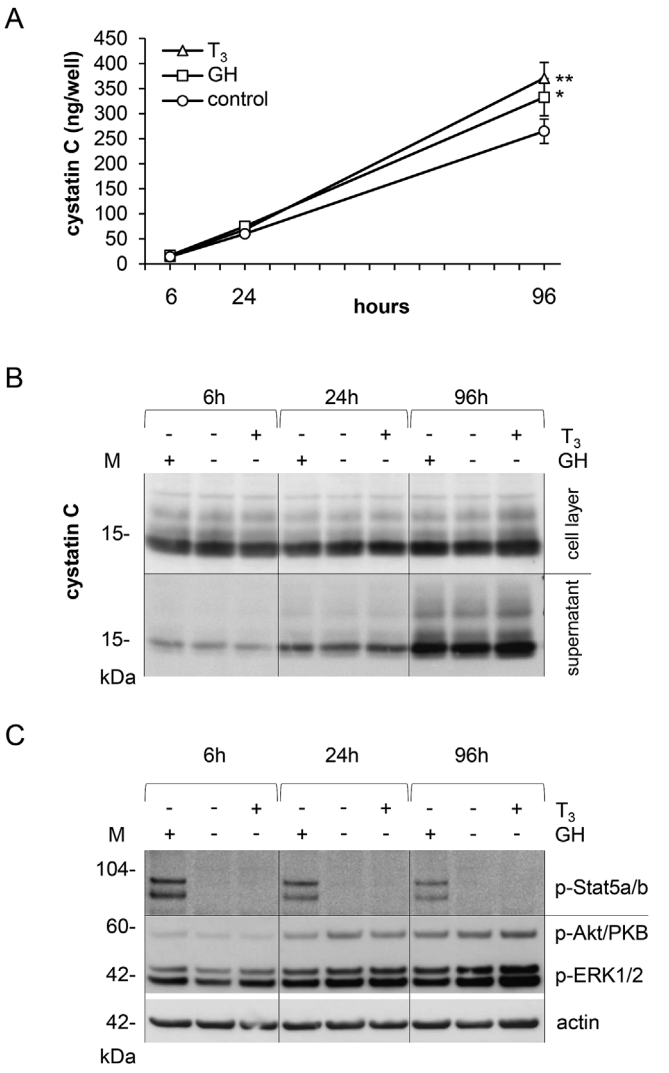


**Fig. 4.** Time-dependent effects of GH and $T_3$ on CysC, p-Stat5a/b, p-Akt/PKB, p-ERK1/2 in 3T3-L1 adipocytes.
Cells were kept on dishes for 11 days and exposed to GH [10 nM] or $T_3$ [10 nM] for 4 d, 1 d, or 6 h prior to analysis as shown in Fig. 1B. **A**: Accumulation of CysC over time in media assessed by ELISA, from 5 experiments carried out in triplicate; *p < 0.05, **p < 0.001. **B**: Representative immunoblot with protein extracts from cell layers (~12% of the lysate, upper panel) and media (~0.6% of the supernatant, lower panel). **C**: Representative immunoblot with protein extracts from cell layers showing phosphorylation of Stat5a/b, Akt/PKB, and ERK1/2; actin as loading control.

*cysC* mRNA and CysC protein was found in adipocytes than in fibroblasts.

Differentiation of fibroblasts (day 3) to adipocytes (day 11) was confirmed by assessment of actin, Pref-1 and PPARγ (1 and 2) expression. After differentiation of our cells from fibroblasts into adipocytes, actin expression was decreased, and Pref-1 was no longer detectable (Fig. 3A). PPARγ 1 and 2 were upregulated, and the abundance of CysC protein was higher in adipocytes than in fibroblasts.

In order to further characterize our 3T3-L1 cells, we also assessed responsiveness to insulin and GH by measuring activation of corresponding signal transduction components and glucose uptake. As shown in Fig. 3B, fibroblasts and adipocytes were both responsive to insulin and GH. As expected, GH increased p-Stat-5a/b and p-ERK1/2, insulin increased p-Akt/PKB and p-ERK1/2. Basal and 1 nM and 100 nM insulin-stimulated 2DG uptake were assessed in 3T3-L1 adipocytes. GH, $T_3$, and dex were included for the last 4 days of culture (according to protocol in Fig. 1A). GH did not significantly affect basal but decreased insulin-dependent 2DG uptake. $T_3$ stimulated basal and insulin-dependent 2DG uptake. Dex tended to increase basal 2DG uptake, but had no significant effect (Table 2).

### 3.3. Effects of GH, $T_3$, and dex on CysC production in 3T3-L1 adipocytes

The production of CysC was studied in 3T3-L1 adipocytes by ELISA and results are shown in Table 3. During 4 days of culture, more CysC accumulated in supernatants (298 ± 10 ng/well) than in cell layers (17.1 ± 1.8 ng/well). Inclusion of $T_3$ [10 nM] during this period significantly increased CysC in cell layers (1.79 ± 0.16 fold) and in supernatants (1.41 ± 0.04 fold). GH [10 nM] increased CysC in supernatants (1.25 ± 0.03 fold). Additional assessment of CysC by immunoblotting essentially confirmed our results. Inclusion of dex [100 nM] also increased the production of CysC (Table 3B), 1.23 ± 0.08 fold in cell layers and 1.22 ± 0.09 fold in supernatants.

Next, time-dependency of CysC production was studied. 3T3-L1 adipocytes (day 11, protocol according to Fig. 1B) were exposed to GH [10 nM] or $T_3$ [10 nM] for 4 d, 1 d, or 6 h prior to analysis. Both GH and $T_3$ increased accumulation of CysC in supernatants over time, as found by ELISA (Fig. 4A) and immunoblotting (Fig. 4B). However, increases in CysC due to exposure to GH and $T_3$ were small and reached significance only after 4 d. Levels of CysC in supernatants after 4 days were far higher than in cell layers: 20 times less of the conditioned supernatants resulted in comparable signal intensities as seen with cell layer lysates (Fig. 4B). These findings with Western blotting agree well with results obtained by ELISA as presented in Table 3. In cell layers, similar levels of CysC were found at 6 h, 1 d, and 4 d. No difference in migration of CysC from cell layers or supernatants was found. As shown in Fig. 4C, inclusion of GH resulted in marked phosphorylation of Stat5 both after shorter and longer exposure times. GH did not affect phosphorylation of Akt/PKB or ERK1/2.

Regulation of CysC production by GH and $T_3$ was dose-dependent (Table 4A). Cells were cultured in the absence or presence of increasing hormone concentrations for 4 d and CysC was determined in culture media by ELISA. 10 nM of GH or at least 1 nM of $T_3$ were required to achieve a significant stimulation of CysC of up to 1.4 fold. Furthermore, GH and $T_3$ when present in combination resulted in an additive increase in CysC (Table 4B).

## 4. Discussion

Analysis of tissue distribution of CysC in mice confirmed high expression in pituitary and brain, and low expression in liver. In addition, adipose tissue depots showed significant expression of CysC. We could also confirm that CysC expression is upregulated during the process of adipocyte differentiation (Kratchmarova et al., 2002; Taleb et al., 2005). In our *in vitro* system, upregulation of CysC was associated with downregulation of the adipogenesis inhibitor pref-1 as well as an increased abundance of differentiation markers PPARγ 1 and 2 (both required for normal adipogenesis), in line with previous reports (Smas and Sul, 1993; Takenaka et al., 2013).

Our results confirm that 3T3-L1 preadipocytes/adipocytes express CysC and release the protein into the media where it accumulates in a time-dependent fashion. CysC production is regulated *in vivo* and *in vitro* by external factors, such as cellular stress and various hormones including insulin at high concentrations (Molina et al., 2009; Zhou et al., 2009; Wang et al., 2006). In response to insulin exposure for 4 days (day 7–11), there was a significant dose-dependent increase of CysC in supernatants (not shown). It is noteworthy that more global, unbiased approaches identified CysC as a protein whose secretion was increased in response to supraphysiological doses of insulin, in rat and mouse adipocytes (Zhou et al., 2009; Wang et al., 2006; Chen et al., 2005).

Both $T_3$ and GH stimulated 3T3-L1 adipocytes to increase the release of CysC into the culture medium. In serum-free cultures, CysC in

**Table 4**
Effects of GH and $T_3$ on CysC accumulation in culture media.

Cells were exposed to GH or $T_3$ at the indicated concentrations (**A**), or to GH, $T_3$, or a combination thereof (**B**) for the last 4 days of culture as shown in Table 3. Media (supernatants) were analysed by ELISA. *$p < 0.05$ for comparison of hormone-treated vs control, **$p < 0.05$ for comparison of combined treatment ($T_3$ + GH) vs GH alone.

A

| hormone [nM] | cystatin C, ng/well x 4 d (treated/control) | |
|---|---|---|
| | GH | $T_3$ |
| - | 318 ± 12 (1) | 322 ± 12 (1) |
| 0.1 | 348 ± 16 (1.09 ± 0.05) | 339 ± 6.0 (1.05 ± 0.02) |
| 1 | 351 ± 8.6 (1.10 ± 0.03) | 387 ± 9.8* (1.20 ± 0.04)* |
| 10 | 453 ± 57* (1.42 ± 0.18)* | 451 ± 5.9* (1.40 ± 0.03)* |

B

| hormone | cystatin C<br>ng/well x 4 d (treated/control) |
|---|---|
| control | 321 ± 10 (1) |
| $T_3$ [10 nM] | 444 ± 11* (1.38 ± 0.03)* |
| GH [10 nM] | 393 ± 10* (1.22 ± 0.03)* |
| $T_3$ + GH | 493 ± 24*/** (1.54 ± 0.08)*/** |

the medium reflects the net release of the protein by the cells. We found a linear increase of CysC in the medium over time (Fig. 4) suggesting that production and secretion by far exceed degradation (or uptake) of the protein in this cell culture system. Culturing the cells under serum-free conditions allowed us to test the effects of $T_3$, GH and insulin without interference by other FCS-contained hormones. To avoid variable residual serum effects, the experiments addressing actions of GH and $T_3$ share a common culture period including the same time in serum-free medium. Furthermore, protein content of cell layers was determined in every experiment, and only wells with similar protein content were used to exclude possible unspecific effects.

Western analysis revealed CysC in cell layers and in supernatants at day 3 and at day 11; in all cases, CysC appeared to have the same molecular size (Figs. 3 and 4). According to our experience, quantification of CysC by ELISA was more precise than semiquantitative estimation by Western blotting. Nevertheless, findings by ELISA and Western analysis were in excellent agreement, and the latter corroborated the identity of the assessed protein and the reliability of our data.

We have studied the regulation of the production of CysC but not its role, which is mainly the local control of proteolysis. In the cells studied, production greatly exceeded disappearance of CysC. *In vivo*, the disappearance of CysC from the circulation predominantly depends on renal glomerular filtration and degradation by kidney tubular epithelial cells although other cells may also internalize the protein. The relative contribution of several organs to circulating levels of CysC is unknown and difficult to estimate. Adipose tissue may contribute significantly to elevated serum CysC as observed in human obesity and hyperinsulinemia. Spillover of CysC into the blood stream is apparently increased in hyperthyroidism and acromegaly, to an extent that serum CysC no longer reflects GFR. $T_3$ stimulates CysC production in two other cell types: in Hep G2 cells (kept in thyroid hormone-stripped medium), as assessed by RT-PCR of the cells and a nephelometric immunoassay of the media, by about 30% (Kotajima et al., 2010), and in preosteoblasts (kept in serum-free medium), as assessed by Northern analysis and analysis of the protein in the media by Western and ELISA, by about 50% (Schmid et al., 2012).

We found that most of the CysC produced by 3T3-L1 adipocytes is released into the media and not retained in the cell layer (Table 3, and Fig. 4). It is striking that 3T3-L1 preadipocytes/adipocytes released about 10 times more CysC than preosteoblasts. In those cells, we hardly could detect CysC in the cell layers by Western analysis, and detection of CysC in supernatants required concentrating the samples (Schmid et al., 2012). Our observation that $T_3$ stimulated CysC production in 3T3-L1 cells confirms and extends previous findings with other cell types. As adipocytes may be a major source of circulating CysC *in vivo*, stimulation by $T_3$ could explain increased serum CysC in hyperthyroidism. Our findings of CysC stimulation by GH are original and need to be confirmed. Stimulatory effects of GH and $T_3$ on CysC *in vivo* are difficult to separate; GH can stimulate production and action of $T_3$ (Goede et al., 2009; Sze et al., 2013; Hussain et al., 1996; Martins et al., 2007). In our *in vitro* experiments, cells cultured in the presence of either GH or $T_3$ alone released more CysC compared to controls, and the combination of the two appeared to have additive rather than synergistic effects.

It has been reported that dex can stimulate CysC gene expression and CysC protein production in tumour cells and more strongly in an osteoblastic cell line (Schmid et al., 2012; Bjarnadottir et al., 1995; Yamawaki et al., 2013). Dex was much less potent in 3T3-L1 cells (Table 3B). In contrast to the effects of GH and $T_3$ ($p < 0.001$), those of dex hardly reached significance. This is in line with *in vivo* findings that showed inconsistent and controversial influence of glucocorticoids on serum CysC (Goede et al., 2009; Bokenkamp et al., 2002).

Thyroid hormones and GH increase cell metabolism; therefore, the demand for proteolysis control may also increase. Similar to oxidative stress and cellular damage, the increased cellular and metabolic turnover in hyperthyroidism and acromegaly is associated with accelerated aging and increased mortality; higher serum levels of CysC in such disease conditions may reflect this. More specifically, $T_3$ stimulation of CysC production by several cells and spillover into the circulation may account for the dependency of CysC serum levels on GH and $T_3$ *in vivo*. Rather than being dominated by renal catabolism and clearance (assuming production rate to be constant), increased plasma CysC in hyperthyroidism and acromegaly (in the face of increased GFR) appear to reflect an important impact of thyroid hormones and GH on the production of CysC.

## Declaration of interest

The authors have no conflicts of interest to declare.

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## References

Abrahamson, M., Olafsson, I., Palsdottir, A., Ulvsback, M., Lundwall, A., Jensson, O., Grubb, A., 1990. Structure and expression of the human cystatin C gene. Biochem. J. 268, 287–294.

Astor, B.C., Shafi, T., Hoogeveen, R.C., Matsushita, K., Ballantyne, C.M., Inker, L.A., Coresh, J., 2012. Novel markers of kidney function as predictors of ESRD, cardiovascular disease, and mortality in the general population. Am. J. Kidney Dis. 59, 653–662.

Ballew, S.H., Chen, Y., Daya, N.R., Godino, J.G., Windham, B.G., McAdams-DeMarco, M., Coresh, J., Selvin, E., Grams, M.E., 2017. Frailty, kidney function, and polypharmacy: the atherosclerosis risk in communities (ARIC) study. Am. J. Kidney Dis. 69, 228–236.

Bjarnadottir, M., Grubb, A., Olafsson, I., 1995. Promoter-mediated, dexamethasone-induced increase in cystatin C production by HeLa cells. Scand. J. Clin. Lab. Invest. 55, 617–623.

Bokenkamp, A., van Wijk, J.A., Lentze, M.J., Stoffel-Wagner, B., 2002. Effect of corticosteroid therapy on serum cystatin C and beta2-microglobulin concentrations. Clin. Chem. 48, 1123–1126.

Chen, X., Cushman, S.W., Pannell, L.K., Hess, S., 2005. Quantitative proteomic analysis of the secretory proteins from rat adipose cells using a 2D liquid chromatography-MS/MS approach. J. Proteome Res. 4, 570–577.

Chew-Harris, J.S., Florkowski, C.M., George, P.M., Elmslie, J.L., Endre, Z.H., 2013. The relative effects of fat versus muscle mass on cystatin C and estimates of renal function in healthy young men. Ann. Clin. Biochem. 50, 39–46.

Codoner-Franch, P., Ballester-Asensio, E., Martinez-Pons, L., Vallecillo-Hernandez, J., Navarro-Ruiz, A., del Valle-Perez, R., 2011. Cystatin C, cardiometabolic risk, and body composition in severely obese children. Pediatr. Nephrol. 26, 301–307.

Cole, T., Dickson, P.W., Esnard, F., Averill, S., Risbridger, G.P., Gauthier, F., Schreiber, G., 1989. The cDNA structure and expression analysis of the genes for the cysteine proteinase inhibitor cystatin C and for beta 2-microglobulin in rat brain. Eur. J. Biochem. 186, 35–42.

de Boer, I.H., Katz, R., Chonchol, M.B., Fried, L.F., Ix, J.H., Kestenbaum, B., Mukamal, K.J., Peralta, C.A., Siscovick, D.S., 2012. Insulin resistance, cystatin C, and mortality among older adults. Diabetes Care 35, 1355–1360.

den Hollander, J.G., Wulkan, R.W., Mantel, M.J., Berghout, A., 2003. Is cystatin C a marker of glomerular filtration rate in thyroid dysfunction? Clin. Chem. 49, 1558–1559.

Donahue, R.P., Stranges, S., Rejman, K., Rafalson, L.B., Dmochowski, J., Trevisan, M., 2007. Elevated cystatin C concentration and progression to pre-diabetes: the Western New York study. Diabetes Care 30, 1724–1729.

Emberson, J.R., Haynes, R., Dasgupta, T., Mafham, M., Landray, M.J., Baigent, C., Clarke, R., 2010. Cystatin C and risk of vascular and nonvascular mortality: a prospective cohort study of older men. J. Intern. Med. 268, 145–154.

Fricker, M., Wiesli, P., Brandle, M., Schwegler, B., Schmid, C., 2003. Impact of thyroid dysfunction on serum cystatin C. Kidney Int. 63, 1944–1947.

Frost, S.C., Lane, M.D., 1985. Evidence for the involvement of vicinal sulfhydryl groups in insulin-activated hexose transport by 3T3-L1 adipocytes. J. Biol. Chem. 260, 2646–2652.

Goede, D.L., Wiesli, P., Brandle, M., Bestmann, L., Bernays, R.L., Zwimpfer, C., Schmid, C., 2009. Effects of thyroxine replacement on serum creatinine and cystatin C in patients with primary and central hypothyroidism. Swiss Med. Wkly. 139, 339–344.

Gosteli-Peter, M.A., Schmid, C., Zapf, J., 1996. Triiodothyronine increases glucose transporter isotype 4 mRNA expression, glucose transport, and glycogen synthesis in adult rat cardiomyocytes in long-term culture. Biochem. Biophys. Res. Commun. 221, 521–524.

Green, H., Meuth, M., 1974. An established pre-adipose cell line and its differentiation in culture. Cell 3, 127–133.

Green, H., Morikawa, M., Nixon, T., 1985. A dual effector theory of growth-hormone action. Differentiation 29, 195–198.

Hakansson, K., Huh, C., Grubb, A., Karlsson, S., Abrahamson, M., 1996. Mouse and rat cystatin C: Escherichia coli production, characterization and tissue distribution. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 114, 303–311.

Hart, A., Blackwell, T.L., Paudel, M.L., Taylor, B.C., Orwoll, E.S., Cawthon, P.M., Ensrud, K.E., Osteoporotic Fractures in Men Study, G, 2017. Cystatin C and the risk of frailty and mortality in older men. J. Gerontol. A Biol. Sci. Med. Sci. 72, 965–970.

Huh, C., Nagle, J.W., Kozak, C.A., Abrahamson, M., Karlsson, S., 1995. Structural organization, expression and chromosomal mapping of the mouse cystatin-C-encoding gene (Cst3). Gene 152, 221–226.

Hussain, M.A., Schmitz, O., Jorgensen, J.O., Christiansen, J.S., Weeke, J., Schmid, C., Froesch, E.R., 1996. Insulin-like growth factor I alters peripheral thyroid hormone metabolism in humans: comparison with growth hormone. Eur. J. Endocrinol. 134, 563–567.

Ix, J.H., Shlipak, M.G., Chertow, G.M., Whooley, M.A., 2007. Association of cystatin C with mortality, cardiovascular events, and incident heart failure among persons with coronary heart disease: data from the Heart and Soul Study. Circulation 115, 173–179.

Jayagopal, V., Keevil, B.G., Atkin, S.L., Jennings, P.E., Kilpatrick, E.S., 2003. Paradoxical changes in cystatin C and serum creatinine in patients with hypo- and hyperthyroidism. Clin. Chem. 49, 680–681.

Jiborn, T., Abrahamson, M., Wallin, H., Malm, J., Lundwall, A., Gadaleanu, V., Abrahamsson, P.A., Bjartell, A., 2004. Cystatin C is highly expressed in the human male reproductive system. J. Androl. 25, 564–572.

Koenig, W., Twardella, D., Brenner, H., Rothenbacher, D., 2005. Plasma concentrations of cystatin C in patients with coronary heart disease and risk for secondary cardiovascular events: more than simply a marker of glomerular filtration rate. Clin. Chem. 51, 321–327.

Kotajima, N., Yanagawa, Y., Aoki, T., Tsunekawa, K., Morimura, T., Ogiwara, T., Nara, M., Murakami, M., 2010. Influence of thyroid hormones and transforming growth factor-beta1 on cystatin C concentrations. J. Int. Med. Res. 38, 1365–1373.

Kratchmarova, I., Kalume, D.E., Blagoev, B., Scherer, P.E., Podtelejnikov, A.V., Molina, H., Bickel, P.E., Andersen, J.S., Fernandez, M.M., Bunkenborg, J., Roepstorff, P., Kristiansen, K., Lodish, H.F., Mann, M., Pandey, A., 2002. A proteomic approach for identification of secreted proteins during the differentiation of 3T3-L1 preadipocytes to adipocytes. Mol. Cell. Proteomics 1, 213–222.

Lee, J.G., Lee, S., Kim, Y.J., Jin, H.K., Cho, B.M., Kim, Y.J., Jeong, D.W., Park, H.J., Kim, J.E., 2009. Multiple biomarkers and their relative contributions to identifying metabolic syndrome. Clin. Chim. Acta 408, 50–55.

Lee, S.H., Park, S.A., Ko, S.H., Yim, H.W., Ahn, Y.B., Yoon, K.H., Cha, B.Y., Son, H.Y., Kwon, H.S., 2010. Insulin resistance and inflammation may have an additional role in the link between cystatin C and cardiovascular disease in type 2 diabetes mellitus patients. Metabolism 59, 241–246.

Levey, A.S., Inker, L.A., Coresh, J., 2014. GFR estimation: from physiology to public health. Am. J. Kidney Dis. 63, 820–834.

Lin, Y., Sun, Z., 2011. Thyroid hormone promotes insulin-induced glucose uptake by enhancing Akt phosphorylation and VAMP2 translocation in 3T3-L1 adipocytes. J. Cell. Physiol. 226, 2625–2632.

Magnusson, M., Hedblad, B., Engstrom, G., Persson, M., Nilsson, P., Melander, O., 2013. High levels of cystatin C predict the metabolic syndrome: the prospective Malmo Diet and Cancer Study. J. Intern. Med. 274, 192–199.

Manetti, L., Pardini, E., Genovesi, M., Campomori, A., Grasso, L., Morselli, L.L., Lupi, I., Pellegrini, G., Bartalena, L., Bogazzi, F., Martino, E., 2005. Thyroid function differently affects serum cystatin C and creatinine concentrations. J. Endocrinol. Invest. 28, 346–349.

Martins, M.R., Doin, F.C., Komatsu, W.R., Barros-Neto, T.L., Moises, V.A., Abucham, J., 2007. Growth hormone replacement improves thyroxine biological effects: implications for management of central hypothyroidism. J. Clin. Endocrinol. Metab. 92, 4144–4153.

Molina, H., Yang, Y., Ruch, T., Kim, J.W., Mortensen, P., Otto, T., Nalli, A., Tang, Q.Q., Lane, M.D., Chaerkady, R., Pandey, A., 2009. Temporal profiling of the adipocyte proteome during differentiation using a five-plex SILAC based strategy. J. Proteome Res. 8, 48–58.

Muntner, P., Winston, J., Uribarri, J., Mann, D., Fox, C.S., 2008. Overweight, obesity, and elevated serum cystatin C levels in adults in the United States. Am. J. Med. 121, 341–348.

Murai, T., Takebe, N., Nagasawa, K., Todate, Y., Nakagawa, R., Nakano, R., Hangai, M., Hasegawa, Y., Takahashi, Y., Yoshioka, K., Ishigaki, Y., 2017. Association of epicardial adipose tissue with serum level of cystatin C in type 2 diabetes. PLoS One 12, e0184723.

Naour, N., Fellahi, S., Renucci, J.F., Poitou, C., Rouault, C., Basdevant, A., Dutour, A., Alessi, M.C., Bastard, J.P., Clement, K., Guerre-Millo, M., 2009. Potential contribution of adipose tissue to elevated serum cystatin C in human obesity. Obesity (Silver Spring) 17, 2121–2126.

Naour, N., Rouault, C., Fellahi, S., Lavoie, M.E., Poitou, C., Keophiphath, M., Eberle, D., Shoelson, S., Rizkalla, S., Bastard, J.P., Rabasa-Lhoret, R., Clement, K., Guerre-Millo, M., 2010. Cathepsins in human obesity: changes in energy balance predominantly affect cathepsin s in adipose tissue and in circulation. J. Clin. Endocrinol. Metab. 95, 1861–1868.

Newman, A.B., Sanders, J.L., Kizer, J.R., Boudreau, R.M., Odden, M.C., Zeki Al Hazzouri, A., Arnold, A.M., 2016. Trajectories of function and biomarkers with age: the CHS all stars study. Int. J. Epidemiol. 45, 1135–1145.

Reutens, A.T., Bonnet, F., Lantieri, O., Roussel, R., Balkau, B., Epidemiological Study on the Insulin Resistance Syndrome Study, G, 2013. The association between cystatin C and incident type 2 diabetes is related to central adiposity. Nephrol. Dial. Transplant. 28, 1820–1829.

Richard, A.J., Stephens, J.M., 2014. The role of JAK-STAT signaling in adipose tissue function. Biochim. Biophys. Acta 1842, 431–439.

Romero, R., Casanova, B., Pulido, N., Suarez, A.I., Rodriguez, E., Rovira, A., 2000. Stimulation of glucose transport by thyroid hormone in 3T3-L1 adipocytes: increased abundance of GLUT1 and GLUT4 glucose transporter proteins. J. Endocrinol. 164, 187–195.

Sahakyan, K., Lee, K.E., Shankar, A., Klein, R., 2011. Serum cystatin C and the incidence of type 2 diabetes mellitus. Diabetologia 54, 1335–1340.

Sarnak, M.J., Katz, R., Fried, L.F., Siscovick, D., Kestenbaum, B., Seliger, S., Rifkin, D., Tracy, R., Newman, A.B., Shlipak, M.G., Cardiovascular Health, S., 2008. Cystatin C and aging success. Arch. Intern. Med. 168, 147–153.

Sasaki-Suzuki, N., Arai, K., Ogata, T., Kasahara, K., Sakoda, H., Chida, K., Asano, T., Pessin, J.E., Hakuno, F., Takahashi, S., 2009. Growth hormone inhibition of glucose uptake in adipocytes occurs without affecting GLUT4 translocation through an

insulin receptor substrate-2-phosphatidylinositol 3-kinase-dependent pathway. J. Biol. Chem. 284, 6061–6070.
Schmid, C., Ghirlanda-Keller, C., Zwimpfer, C., Zoidis, E., 2012. Triiodothyronine stimulates cystatin C production in bone cells. Biochem. Biophys. Res. Commun. 419, 425–430.
Servais, A., Giral, P., Bernard, M., Bruckert, E., Deray, G., Isnard Bagnis, C., 2008. Is serum cystatin-C a reliable marker for metabolic syndrome? Am. J. Med. 121, 426–432.
Shankar, A., Tep. Clin. Hypertens (Greenwich)pala, S., 2011. Relationship between body mass index and high cystatin levels among US adults. J. Clin. Hypertens (Greenwich). 13, 925–930.
Shlipak, M.G., Sarnak, M.J., Katz, R., Fried, L.F., Seliger, S.L., Newman, A.B., Siscovick, D.S., Stehman-Breen, C., 2005. Cystatin C and the risk of death and cardiovascular events among elderly persons. N. Engl. J. Med. 352, 2049–2060.
Simonsen, O., Grubb, A., Thysell, H., 1985. The blood serum concentration of cystatin C (gamma-trace) as a measure of the glomerular filtration rate. Scand. J. Clin. Lab. Invest. 45, 97–101.
Smas, C.M., Sul, H.S., 1993. Pref-1, a protein containing EGF-like repeats, inhibits adipocyte differentiation. Cell 73, 725–734.
Stevens, L.A., Schmid, C.H., Greene, T., Li, L., Beck, G.J., Joffe, M.M., Froissart, M., Kusek, J.W., Zhang, Y.L., Coresh, J., Levey, A.S., 2009. Factors other than glomerular filtration rate affect serum cystatin C levels. Kidney Int. 75, 652–660.
Stewart, W.C., Baugh Jr., J.E., Floyd, Z.E., Stephens, J.M., 2004. STAT 5 activators can replace the requirement of FBS in the adipogenesis of 3T3-L1 cells. Biochem. Biophys. Res. Commun. 324, 355–359.
Surendar, J., Indulekha, K., Aravindhan, V., Ganesan, A., Mohan, V., 2010. Association of cystatin-C with metabolic syndrome in normal glucose-tolerant subjects (CURES-97). Diabetes Technol. Ther. 12, 907–912.
Sze, L., Bernays, R.L., Zwimpfer, C., Wiesli, P., Brandle, M., Schmid, C., 2013. Impact of growth hormone on cystatin C. Nephron Extra 3, 118–124.
Takano, A., Haruta, T., Iwata, M., Usui, I., Uno, T., Kawahara, J., Ueno, E., Sasaoka, T., Kobayashi, M., 2001. Growth hormone induces cellular insulin resistance by uncoupling phosphatidylinositol 3-kinase and its downstream signals in 3T3-L1 adipocytes. Diabetes 50, 1891–1900.
Takenaka, Y., Inoue, I., Nakano, T., Shinoda, Y., Ikeda, M., Awata, T., Katayama, S., 2013. A Novel Splicing Variant of Peroxisome Proliferator-Activated Receptor-gamma (Ppargamma1sv) Cooperatively Regulates Adipocyte Differentiation with Ppargamma2. PLoS One 8, e65583.
Taleb, S., Lacasa, D., Bastard, J.P., Poitou, C., Cancello, R., Pelloux, V., Viguerie, N., Benis, A., Zucker, J.D., Bouillot, J.L., Coussieu, C., Basdevant, A., Langin, D., Clement, K., 2005. Cathepsin S, a novel biomarker of adiposity: relevance to atherogenesis. Faseb. J. 19, 1540–1542.
Tangri, N., Inker, L.A., Tighiouart, H., Sorensen, E., Menon, V., Beck, G., Shlipak, M., Coresh, J., Levey, A.S., Sarnak, M.J., 2012. Filtration markers may have prognostic value independent of glomerular filtration rate. J. Am. Soc. Nephrol. 23, 351–359.
Uruska, A., Araszkiewicz, A., Zozulinska-Ziolkiewicz, D., Wegner, M., Grzelka, A., Wierusz-Wysocka, B., 2014. Does serum cystatin C level reflect insulin resistance in patients with type 1 diabetes? Clin. Biochem. 47, 1235–1238.
Vigil, L., Lopez, M., Condes, E., Varela, M., Lorence, D., Garcia-Carretero, R., Ruiz, J., 2009. Cystatin C is associated with the metabolic syndrome and other cardiovascular risk factors in a hypertensive population. J. Am. Soc. Hypertens. 3, 201–209.
Wang, P., Keijer, J., Bunschoten, A., Bouwman, F., Renes, J., Mariman, E., 2006. Insulin modulates the secretion of proteins from mature 3T3-L1 adipocytes: a role for transcriptional regulation of processing. Diabetologia 49, 2453–2462.
Weinstein, S.P., Watts, J., Graves, P.N., Haber, R.S., 1990. Stimulation of glucose transport by thyroid hormone in ARL 15 cells: increased abundance of glucose transporter protein and messenger ribonucleic acid. Endocrinology 126, 1421–1429.
Wiesli, P., Schwegler, B., Spinas, G.A., Schmid, C., 2003. Serum cystatin C is sensitive to small changes in thyroid function. Clin. Chim. Acta 338, 87–90.
Woitas, R.P., Kleber, M.E., Meinitzer, A., Grammer, T.B., Silbernagel, G., Pilz, S., Tomaschitz, A., Weihrauch, G., Dobnig, H., Marz, W., Scharnagl, H., 2013. Cystatin C is independently associated with total and cardiovascular mortality in individuals undergoing coronary angiography. The Ludwigshafen Risk and Cardiovascular Health (LURIC) study. Atherosclerosis 229, 541–548.
Yamashita, H., Nishino, T., Obata, Y., Nakazato, M., Inoue, K., Furusu, A., Takamura, N., Maeda, T., Ozono, Y., Kohno, S., 2013. Association between cystatin C and arteriosclerosis in the absence of chronic kidney disease. J. Atherosclerosis Thromb. 20, 548–556.
Yamawaki, C., Takahashi, M., Takara, K., Kume, M., Hirai, M., Yasui, H., Nakamura, T., 2013. Effect of dexamethasone on extracellular secretion of cystatin C in cancer cell lines. Biomed. Rep. 1, 115–118.
Young, J.A., Hwang, S.J., Sarnak, M.J., Hoffmann, U., Massaro, J.M., Levy, D., Benjamin, E.J., Larson, M.G., Vasan, R.S., O'Donnell, C.J., Fox, C.S., 2008. Association of visceral and subcutaneous adiposity with kidney function. Clin. J. Am. Soc. Nephrol. 3, 1786–1791.
Zhou, H., Xiao, Y., Li, R., Hong, S., Li, S., Wang, L., Zeng, R., Liao, K., 2009. Quantitative analysis of secretome from adipocytes regulated by insulin. Acta Biochim. Biophys. Sin. (Shanghai) 41, 910–921.
Zoidis, E., Ghirlanda-Keller, C., Schmid, C., 2012. Triiodothyronine stimulates glucose transport in bone cells. Endocrine 41, 501–511.